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Stereospecificity in algal oxidation of the carcinogen benzo(a)pyrene

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Summary. The green alga *Selenastrum capricornutum* Printz metabolizes benzo(a)pyrene to vicinal dihydrodiols. Two metabolites isolated from algal medium extracts have been identified as *cis*-4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene and *cis*-7,8-dihydroxy-7,8-dihydro-benzo(a)pyrene. No *trans*-dihydrodiols have been found. This stereospecificity is unlike that seen in fungal and mammalian systems, in which polycyclic aromatic compounds are metabolized to *trans*-dihydrodiols.
Key words. Benzo(a)pyrene; green algae; *cis*-dihydrodiol; high performance liquid chromatography; fluorescence spectroscopy.

Polycyclic aromatic hydrocarbons (PAHs) are formed during the incomplete combustion of carbonaceous materials, and are widely dispersed into the environment by waste burning, fuel exhaust, and various industrial processes[2]. Interest in this class of chemicals stems from the fact that many are mutagens and carcinogens in mammalian systems[3-5]. In order to produce their mutagenic or carcinogenic effects, PAHs must first be metabolized[6]. As widespread environmental pollutants, these compounds are susceptible to microbial metabolism as well as to metabolism by higher plants and animals. In mammals PAHs are oxidized by a cytochrome P-450 catalyzed monooxygenase to arene oxides which can then be enzymatically hydrated to *trans*-dihydrodiols[7-9]. Certain *trans*-dihydrodiols may be further oxidzed to dihydrodiol-epoxides. For certain PAHs, including benzo(a)pyrene (BaP)[6], specific dihydrodiol-epoxides are generally regarded as being ultimate carcinogenic metabolites. Fungi have also been shown to metabolize PAHs to *trans*-dihydrodiols by a monooxygenase reaction[10]. In contrast to fungal and mammalian systems, PAHs are metabolized by bacteria to *cis*-dihydrodiols *via* a dioxygenase enzyme system[10].

Few studies have dealt with metabolism of PAHs in photosynthetic organisms[11,12]. We recently reported that BaP, a potent carcinogenic PAH, is metabolized to dihydrodiols by green algal cultures[13]. The present study was undertaken to determine the stereochemistry of BaP dihydrodiols formed by *Selenastrum capricornutum* Printz, a freshwater planktonic green alga. We determined that the 4,5-and 7,8-dihydrodiols are *cis* isomers using a combination of fluorescence spectroscopy, high performance liquid chromatography (HPLC), and liquid scintillation spectrometry.

Cultures of *S. capricornutum* (strain no. UTEX 1648) obtained from the University of Texas in Austin were maintained in a synthetic nutrient medium[14]. Algal cultures were aseptically dispensed into 125-ml Delong flasks at a density of 5×10^5 cells/ml in 25 ml of medium per flask. A mixture containing ^{14}C-labeled (0.5 μCi) and unlabeled BaP (a total of 30 μg) dissolved in 100 μl ethylene glycol monomethyl ether (EGME) was added to each flask. Neither EGME nor the dose of BaP administered had a significant effect on growth. Algal cultures were incubated on a New Brunswick Scientific gyrotory shaker at 100 rpm in a Percival incubator at 23 °C. Cultures were illuminated during incubation with gold fluorescent lamps (G.E. F20T12/GO) on a 24-h light/dark cycle (16 h light, 8 h dark). Photooxidation of BaP is negligible and algal growth is sustained under these conditions. Cultures were routinely monitored for bacterial, fungal, or yeast contamination and no contamination was detected in the cultures used in this study.

After a four-day incubation period, the algal suspension was centrifuged at 1500 rpm for 5 min at 5 °C. BaP and its metabolites, 90% of which were found in the medium, were recovered by extraction of the medium with ethyl acetate (3 × 12.5 ml). The extracts were pooled, evaporated to dryness under nitrogen, and stored at −20 °C. Samples were brought to room temperature just prior to HPLC analysis.

The 4,5- and 7,8-dihydrodiols were isolated from the algal medium extract by reversed-phase HPLC as previously described[15]. The percent conversion of BaP to these dihydrodiols was 2.2% and less than 1% for the 7,8- and 4,5-dihydrodiols, respectively. These dihydrodiols were not found in control flasks containing only BaP and algal medium. In order to determine the stereochemistry of each dihydrodiol, HPLC methods were developed using synthetic standards[16] to separate *cis* and *trans* isomers of these two dihydrodiols. All separations were performed at room temperature on a Waters liquid chromatographic system equipped with a Whatman Partsil 10 ODS-2 reversed-phase column with a flow of 1 ml/min. Runs were monitored at 254 nm

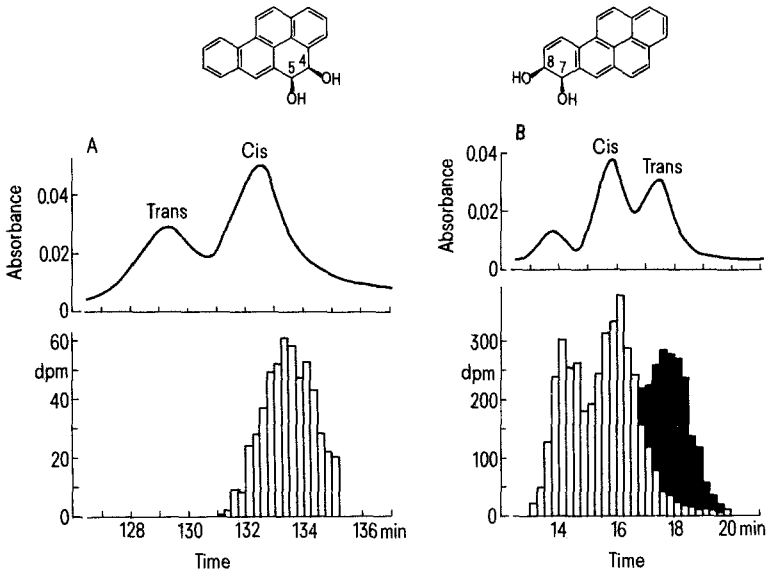

Figure 1. HPLC chromatographs of standard *cis*- and *trans*-dihydodiols cochromatographed with (A) 4,5- and (B) 7,8-dihydrodiols isolated from algal cultures incubated with ^{14}C-BaP. Open bars represent radioactivity extracted from algal medium. Closed bars represent addition of synthetic ^{14}C-*trans*-7,8-dihydrodiol BaP spike (1500 dpm) to the medium extract.

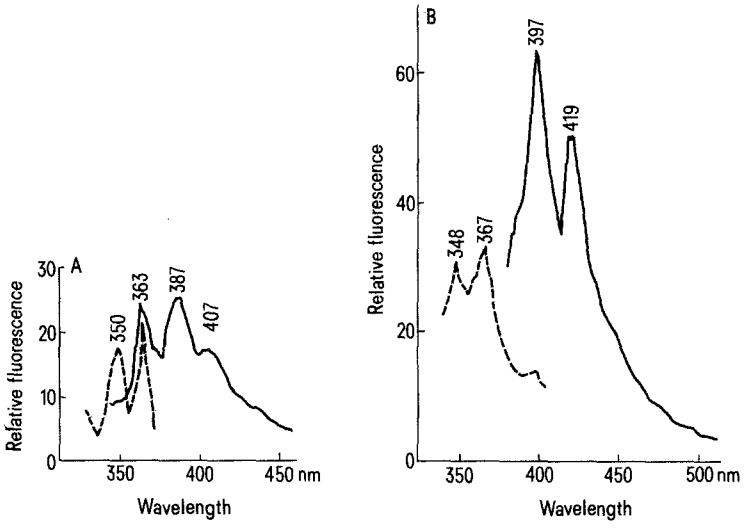

Figure 2. Fluorescence excitation (---) and emission (–) spectra of (A) 4,5- and (B) 7,8-dihydrodiols of BaP isolated from algal medium extract. Only the long wavelengths of the excitation spectra are reported.

on a Waters Model 440 UV absorbance detector. Fractions (0.25 ml) were collected with an ISCO Model 211 automatic fraction collector and radioactivity was quantitated by scintillation counting using a Packard Tri Carb Liquid Scintillation Counter Model 460CD. Separation of the *cis*- and *trans*-4,5-dihydrodiols was achieved with a methanol:water gradient elution program (initial, 50% methanol; linear increase to 70% methanol between 115 and 135 min). The 4,5-dihydrodiol isolated from the algal medium was reconstituted in 40 µl chloroform. A sample containing 10 µl of this solution plus synthetic (unlabeled) *cis*- and *trans*-4,5-dihydrodiol standards was injected onto the liquid chromatographic system described. The 7,8-dihydrodiol isolated from the algal medium was reconstituted and injected onto the HPLC in the same fashion, using synthetic (unlabeled) *cis*- and *trans*-7,8-dihydrodiol standards. These dihydrodiols were separated using a different methanol:water gradient elution program (initial, 72% methanol; linear increase to 76% methanol between 10 and 14 min; linear increase to 80% methanol between 14 and 16 min; linear increase to 92% methanol between 16 and 19 min).

The ^{14}C-4,5-dihydrodiol produced by the algae eluted from the HPLC with similar retention time to the cis-4,5-dihydrodiol standard (fig. 1A). Likewise, the ^{14}C-7,8-dihydrodiol produced by the algae cochromatographed with the *cis*-7,8-dihydrodiol standard (fig. 1B). An additional radiolabeled peak eluted just prior to the *cis*-7,8 standard (fig. 1B). This peak is unidentified as yet, but is possibly another dihydrodiol of BaP. The absence of *trans*-7,8-dihydrodiol was validated by the addition of a ^{14}C-labeled *trans*-7,8-dihydrodiol spike to the algal medium extract. This resulted in the appearance of a third peak of radioactivity which eluted with the unlabeled *trans* standard as would be expected (fig. 1B, closed bars).

Fluorescence spectroscopy was used for structural verification of positional isomers. Dihydrodiols isolated from the algal medium were chromatographed without the unlabeled standards to

obtain fluorescence spectra of the algal products. Fractions were collected in the time intervals which corresponded to the elution times for the standards. Fluorescence spectra were recorded on collected fractions with an Aminco-Bowman spectrophotofluorometer that corrects for instrumentation artifacts including lamp output, photomultiplier response and monochromator grating. Collected fractions which corresponded to peak retention times of the *trans*-4,5- and *trans*-7,8-dihydrodiol standards had no detectable fluorescence. Fractions corresponding to peak retention times of the *cis*-4,5- and *cis*-7,8-dihydrodiol standards had fluorescence spectra with identical excitation and emission maxima to the synthetic 4,5- and 7,8-dihydrodiol standards, respectively (fig. 2). It should be noted that while fluorescence spectroscopy can distinguish positional BaP isomers, this method cannot distinguish between the *cis* and *trans* isomers of these dihydrodiols. However, the combination of cochromatography and fluorescence spectroscopy has allowed the identification of these metabolites as *cis*-dihydrodiols.

The metabolism of BaP to *cis*-dihydrodiols by a green alga suggests that this eukaryotic microorganism metabolizes PAHs *via* a dioxygenase system similar to that observed in prokaryotes. However, formation of *cis*-dihydrodiols by *cis*-hydration of epoxides is a feasible alternative. Such a mechanism has been reported for hydration of an aliphatic epoxide in a fungus, *Fusarium solani pisi*[17]. The stereospecificity reported here for a green alga is significant from an evolutionary standpoint in that the green algae are generally regarded as being the progenitors of the higher plants. Studies are ongoing in our laboratory to determine whether the higher orders of green plants metabolize PAHs to *cis*-dihydrodiols, as does the green alga *S. capricornutum*, or whether they have acquired a monooxygenase enzyme system similar to that of fungi and mammals.

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Photodecomposition of orellanine and orellinine, the fungal toxins of *Cortinarius orellanus* Fries and *Cortinarius speciossimus*

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Summary. The photosensitivity of orellanine, the main toxin of *Cortinarius orellanus* Fries mushrooms, and its transformation to orelline via orellinine is discussed. All three substances were found in methanolic extracts of *Cortinarius orellanus* and *Cortinarius speciossimus* mushrooms. The problem of homogeneity of orellanine is also discussed.
Key words. Orellanine; orellinine; orelline; *Cortinarius orellanus, Cortinarius speciossimus.*

Research on the toxins present in *Cortinarius orellanus* Fries was initiated by Grzymala[1], who isolated a crystalline, colorless substance, and called it orellanine. Orellanine had toxic effects in humans, and also in cats, mice and guinea pigs, and caused histopathological changes in kidneys, liver and spleen identical with those caused by the feeding of intact fungi or their methanolic extract. Grzymala found that orellanine underwent rapid explosive decomposition when heated to about 270 °C, yielding a yellow, sublimable product which was nontoxic. Years later, research on toxins of *Cortinarius orellanus* was undertaken by other authors[2-10].

Paper chromatography of the ethanol extract of the mushrooms, performed by Gruber[2], showed the presence of some undefined, fluorescing substances. Testa[3], using preparative TLC on silica gel and a weakly polar developing system (cyclohexane-ethyl acetate 3:1), isolated four substances from the methanol extract of the fungus, and named them: grzymaline, benzonine 'a' and 'b', and cortinarine, respectively. Testa suggested that these isolated substances were the components of Grzymala's orellanine, which, according to him, was a heterogenous material. Gramper[4] and Moser[5,6] came to similar conclusions. Moser and Kürnsteiner[6], using gel filtration on Sephadex LH20, isolated a substance which showed great similarity in spectral data, identical biological activity, and sensitivity to UV radiation, with the orellanine isolated by Grzymala and by us. The substance isolated by these authors differed from orellanine in its solubility in water; orellanine was soluble only in aqueous alkali, and not in neutral and weakly acidic solutions. Besides this 'slow-action toxin', the presence of another toxin in the methanol extract of the fungus was discovered[6]. The second toxin, in contrast to the first one, showed rapid (observable) onset of biological activity. Using the improved Grzymala's procedure we isolated[7,9] from *Cortinarius orellanus* mushrooms the toxic substance, which showed after purification chromatographic homogeneity and